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FERREDOXIN AS A REDUCTANT IN PYRUVATE SYNTHESIS BY A BACTERIAL EXTRACT

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It is now well established that reduced pyridine nucleotides supply the hydrogen (electrons) required for the reductive steps in the cellular synthesis of carbon compounds. The oxidation-reduction potential of pyridine nucleotides (TPN or DPN) is −320 mv at pH 7. When Tagawa and Arnon[1] determined that the oxidation-reduction potential of ferredoxins isolated from spinach chloroplasts and *Clostridium pasteurianum* is about 100 mv more electronegative than that of pyridine nucleotides, it became a matter of conjecture whether ferredoxins can participate directly as reductants in carbon assimilation. Their indirect participation by way of pyridine nucleotides, with an attendant drop of about 100 mv in reducing potential, was not in doubt because ferredoxins are known to act as electron carriers in the reduction of TPN by illuminated chloroplasts (see review[2]) and by cell-free bacterial extracts.[3] However, there was no experimental evidence for the direct participation of ferredoxin as a reductant in any enzymic reaction concerned with carbon assimilation.

Evidence has now been obtained that ferredoxin (in reduced form) is required for the reductive synthesis of pyruvate from CO_2 and acetyl phosphate by a cell-free extract of *Clostridium pasteurianum* (eq. 1).

$$CO_2 + \text{acetyl phosphate} + \text{ferredoxin}_{red} \xrightarrow{CoA} \text{pyruvate} + \text{ferredoxin}_{oxid} \quad (1)$$

The "phosphoroclastic" degradation of pyruvate (in the presence of phosphate) to acetyl phosphate, CO_2, and H_2 by cell-free extracts of *C. butylicum* was first described by Koepsell, Johnson, and Meek.[4] The cofactor requirements of this reaction were found by Wolfe and O'Kane[5] to include coenzyme A, thiamine pyrophosphate, and a divalent metal. They also noted that the phosphoroclastic degradation of pyruvate differed from other oxidative decarboxylations in showing no requirement for pyridine nucleotide.

A further study of the cofactors of the phosphoroclastic degradation of pyruvate reaction in *C. pasteurianum* led Mortenson, Valentine, and Carnahan[6] to the isolation of ferredoxin as an electron-transferring protein which couples pyruvic dehydrogenase with hydrogenase and leads to the formation of H_2.

The reversibility of the phosphoroclastic degradation of pyruvate was first indicated by Wilson, Krampitz, and Werkman,[7] who observed a rapid exchange of CO_2 with the carboxyl group of pyruvate. Wolfe and O'Kane[8] found that the cofactor requirements of the CO_2 exchange reaction were the same as those of the pyruvate degradation reaction and, more recently, Whiteley and McCormick[9] found that ferredoxin stimulated the CO_2 exchange reaction in *Micrococcus lactilyticus*.

Unlike the CO_2 exchange, the incorporation of acetyl phosphate into pyruvate was more difficult to demonstrate. Mortlock and Wolfe[10] found that in extracts of *C. butylicum* the incorporation of acetyl phosphate into pyruvate depended on sufficient reducing potential, which they provided by adding to the reaction mixture a strong, nonphysiological, reducing agent, sodium hydrosulfite. In the present experiments with extracts of *C. pasteurianum*, the reducing potential required for the synthesis of pyruvate from acetyl phosphate and CO_2 was supplied by the native protein, ferredoxin.

Methods.—Cell-free extracts of *C. pasteurianum* were prepared by sonic oscillation from a paste of frozen cells, essentially as described by Buchanan, Lovenberg, and Rabinowitz.[11] The extracts contained ferredoxin and the enzymes necessary for pyruvate synthesis but these were, in general, unstable. In certain cases, the capacity for pyruvate synthesis was lost completely after storage in air for 6 hr at 4°. To remove ferredoxin, the extract was passed through a DEAE-cellulose column, as described previously.[11] The enzymes necessary for pyruvate synthesis were not retained on the column and were used without further treatment.

The ferredoxin used was isolated from *C. pasteurianum* and crystallized by the methods of Tagawa and Arnon.[1] Reduced ferredoxin was supplied either by reducing oxidized ferredoxin with hydrogen gas and the hydrogenase present in the bacterial extracts or with illuminated spinach chloroplast fragments.

The washed spinach chloroplast fragments (P_1S_1), which were free of ferredoxin, were prepared according to Whatley and Arnon[12] and were heated for 5 min at 55° prior to use. The heat treatment destroyed their capacity for oxygen evolution[13] but did not abolish their capacity to use ascorbate (plus catalytic amounts of dichlorophenol indophenol) as the electron donor system for the photoreduction of ferredoxin.

The enzyme reactions were carried out in Warburg vessels, equilibrated with the desired gas. Hydrogen evolution was measured, where indicated, by the Warburg manometric technique. Following the incubation period, the reaction was stopped by adding 0.1 ml of 70 per cent perchloric acid. Denatured protein was removed by centrifugation. One hundred μmoles of carrier sodium pyruvate were then added to 1.0 ml of the supernatant solution containing the newly synthesized labeled pyruvate. The 2,4-dinitrophenylhydrazone derivative of pyruvate was then prepared as described by Rabinowitz.[14]

Total pyruvate formed from $C^{14}O_2$ and acetyl phosphate was determined by pipetting suitable aliquots of the isolated 2,4-dinitrophenylhydrazone on plastic planchets, drying under a heat lamp, and counting radioactivity with a thin-window

TABLE 1

FERREDOXIN-DEPENDENT SYNTHESIS OF PYRUVATE FROM $C^{14}O_2$, H_2, AND ACETYL PHOSPHATE

Treatment	$C^{14}O_2$ fixed as pyruvate (cpm)
Complete	16,400
Acetyl phosphate omitted*	81
H_2 omitted†	126
Ferredoxin omitted	2,565
Coenzyme A omitted	10,110

* Acetate as a substitute for acetyl phosphate gave 418 cpm.

† H_2 was replaced by N_2. Argon or air was eqaully ineffective as a substitute for H_2.

The complete system contained cell-free extract of *C. pasteurianum* (2.8 mg protein), 100 μg crystalline ferredoxin, and the following in μmoles: potassium phosphate buffer, pH 7.3, 300; acetyl phosphate (lithium salt), 50; coenzyme A, 0.5; and carbon-14 bicarbonate, 10. Final volume, 3.0 ml. Gas phase was hydrogen, except as indicated. The reaction was carried out at 30°C for 30 min.

conventional Geiger-Mueller counter. Eleven thousand cpm corresponded to 0.1 μmole $C^{14}O_2$ fixed as pyruvate. Protein concentrations were estimated by the phenol method as modified by Rabinowitz and Pricer.[15]

Results and Discussion.—Table 1 shows that ferredoxin was required for the synthesis of pyruvate from acetyl phosphate, $C^{14}O_2$, and H_2 by the bacterial extract. No significant formation of pyruvate occurred without hydrogen gas or without added acetyl phosphate. Pyruvate was identified as the 2,4-dinitrophenylhydrazone derivative by paper chromatography with tertiary amyl alcohol-ethanol-water solvent systems.[16, 17] With both solvent systems, radioautography and sectional counting established the coincidence of the 2,4-dinitrophenylhydrazone spot obtained with authentic pyruvate, with the radioactive 2,4-dinitrophenylhydrazone of pyruvate isolated from the reaction mixture.

A requirement for ferredoxin for pyruvate formation was demonstrated only after the endogenous ferredoxin was largely removed by passing the bacterial extract through a DEAE-cellulose column. The untreated bacterial extract gave the same total synthesis of pyruvate but showed no response to the addition of ferredoxin. The untreated extract also gave no response to the addition of coenzyme A, which was shown by Wolfe and O'Kane to be required for the degradation of pyruvate and for the CO_2 exchange reaction.[5, 8]

In the experiments represented by Table 1, hydrogen gas and the native bacterial hydrogenase jointly constituted the electron donor system for ferredoxin. Table 2 is typical of experiments in which the hydrogen gas-hydrogenase electron donor system was replaced by a photochemical electron donor system, consisting of heated spinach chloroplasts and ascorbate-indophenol dye, which was used by Paneque and Arnon[18] to demonstrate photoproduction of hydrogen gas by illuminated chloroplasts. The photochemical electron donor system was more effective than the H_2-hydrogenase system in maintaining, under our experimental conditions, a high level of reduced ferredoxin and gave, therefore, a higher rate of pyruvate synthesis (Table 2).

In the experiments represented in Table 1, hydrogenase was necessary for the reduction of ferredoxin by H_2. But in the photochemical system (Table 2) pyruvate synthesis was most active when hydrogenase was inhibited. As shown in Table 3, the highest rates of pyruvate formation were obtained in the presence of carbon monoxide, which inhibited hydrogenase.[19] When carbon monoxide was replaced by argon, pyruvate formation was greatly decreased and a vigorous production of hydrogen gas ensued. Thus, in an experiment parallel to that shown in Table 3, the photoproduction of H_2 by illuminated chloroplasts was 8.6 μmoles in an atmosphere of argon and 1.1 μmoles in an atmosphere of carbon monoxide. No pyruvate was formed nor was H_2 evolved in the presence of air.

TABLE 2

PHOTOREDUCED FERREDOXIN IN THE REDUCTIVE SYNTHESIS PYRUVATE FROM $C^{14}O_2$ AND ACETYL PHOSPHATE

Treatment	$C^{14}O_2$ fixed as pyruvate (cpm)
Complete	83,500
Acetyl phosphate omitted	795
Ferredoxin omitted	2,300
CoA omitted	16,300

The complete system contained cell-free extract of *C. pasteurianum* (4.3 mg protein), 100 μg crystalline ferredoxin, heated chloroplast fragments equivalent to 0.5 mg chlorophyll, and the following in μmoles: potassium phosphate buffer, pH 7.3, 300; $MgCl_2$, 5; dichlorophenol indophenol, 0.2; sodium ascorbate, 20; acetyl phosphate (lithium salt), 50; coenzyme A, 0.5; and carbon-14 bicarbonate, 10. Light intensity was 10,000 lux. Gas phase was carbon monoxide. Other experimental conditions were as described in Table 1.

TABLE 3

EFFECT OF THE GAS PHASE ON THE REDUCTIVE SYNTHESIS OF PYRUVATE IN THE LIGHT

Gas phase	$C^{14}O_2$ fixed as pyruvate (cpm)
Carbon monoxide	65,000
Hydrogen	29,800
Argon	3,680
Nitrogen	2,700
Air	162

The reaction was carried out under the indicated gas phase. Other experimental conditions were as described in Table 2.

These results indicate that, under the conditions of the experiments, the photoproduction of hydrogen gas by illuminated chloroplasts was competing with the pyruvate-forming reaction. Vigorous pyruvate formation occurred when photoproduction of hydrogen gas by the chloroplast-hydrogenase system was inhibited by an atmosphere of carbon monoxide or by an atmosphere of H_2.[20]

Figure 1 shows the time course of pyruvate synthesis by the photochemical system, and Figure 2 the dependence of the reaction on the concentration of ferredoxin. The reaction proceeded almost linearly for about half an hour, and the rate was proportional to ferredoxin concentration. The highest rates of pyruvate formation were obtained with ferredoxin from *C. pasteurianum*, but ferredoxins from either spinach chloroplasts or the photosynthetic sulfur bacterium, *Chroma-*

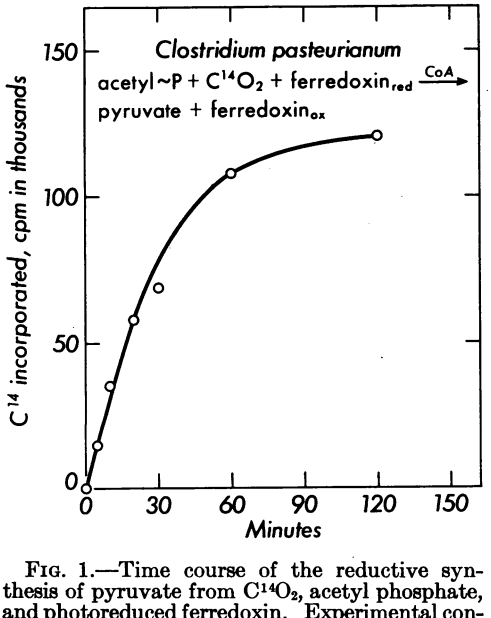

FIG. 1.—Time course of the reductive synthesis of pyruvate from $C^{14}O_2$, acetyl phosphate, and photoreduced ferredoxin. Experimental conditions as described in Table 2.

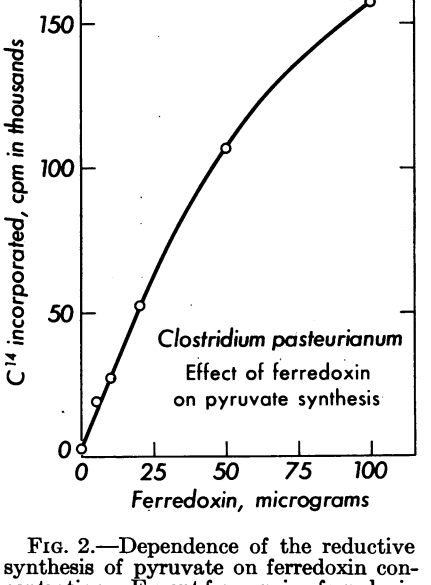

FIG. 2.—Dependence of the reductive synthesis of pyruvate on ferredoxin concentration. Except for varying ferredoxin concentration, experimental conditions were as described in Table 2.

tium, were also effective, whether reduced by hydrogen gas in the dark or photochemically. Neither the addition of reduced TPN and DPN nor the addition of thiamine pyrophosphate and divalent metals had a significant effect on the reductive synthesis of pyruvate by the DEAE-treated bacterial extracts.

As already stated, ferredoxin was shown previously to stimulate the pyruvate-CO_2 exchange reaction in extracts of *M. lactilyticus*.[9] The present experiments with extracts of *C. pasteurianum* provide direct evidence that ferredoxin can act as a hydrogen or electron carrier in a net synthesis of pyruvate from CO_2 and acetyl phosphate. Experiments are now under way to determine whether the strong reducing potential of ferredoxin can also serve directly, without the mediation of pyridine nucleotides, in the reductive carbon assimilation of photosynthesis.

Summary.—Extracts of *Clostridium pasteurianum*, when supplied with reduced ferredoxin, catalyzed a reductive synthesis of pyruvate from carbon dioxide and acetyl phosphate. Reduced ferredoxin was prepared either by reducing the oxidized form with hydrogen gas and hydrogenase in the dark, or photochemically, by illuminated chloroplast fragments.

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